Inhibition of Leukocyte-Mediated Tissue Destruction by Synthetic Fibronectin Peptide (Trp-9-Tyr)

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Burns are surrounded by an inflammatory zone of stasis that can progress to ischemia and extension of burn size. Synthetic fibronectin peptides have reduced tissue destruction in several models of inflammation. In this study, we postulate that administration of the peptide Trp-9-Tyr will alter the progression of tissue destruction following thermal injury. Baseline cutaneous blood flow was measured on New Zealand White rabbits with a laser doppler blood-flow meter. While the rabbits were under general anesthesia, 6 full-thickness burns were produced on the rabbits' backs. Blood flow in the zones of stasis was followed daily, and the number of zones that progressed to necrosis was determined at 72 hours. There were 3 experimental groups. Ten control animals received saline. Ten were treated with Trp-9-Tyr for 24 hours postburn. Ten received Trp-9-Tyr for 48 hours. Animals treated with Trp-9-Tyr had higher blood flow and less necrosis in the zones of stasis than did control animals, which was evident at 24 hours but more significant at 48 hours. (J Burn Care Rehabil 2000;20:505-10)

Thermal injury produces an area of tissue destruction surrounded by a zone of stasis that can progress to ischemia and result in extension of burn size.[1] Leukocytes have been implicated in the pathogenesis and progression of microvascular injury and extravascular tissue damage. Leukocyte-mediated injury is partly dependent on polymorphonuclear neutrophil adherence to endothelial cell surface and leukocyte aggregation in extracellular tissue.[2]

Leukocyte adherence to endothelial cells and to extracellular matrix components is mediated by multiple-adhesion receptor systems. In the first stages of inflammation, leukocyte rolling into inflammatory sites is mediated by the selectin family of adhesion receptors.[3] Additional cellular-recognition receptors, the integrins, then mediate leukocyte binding to the endothelium.[4] Once leukocytes migrate across the endothelial cell membrane, their adherence to the extracellular matrix is directed by many factors, including integrins. Integrins consist of 2 membrane glycoproteins, a larger α subunit and a smaller β subunit. The β_2 subunits are largely involved in cell-to-cell interactions, whereas β_1 subunits are associated with mediating cell adhesion to extracellular matrix constituents.[5]

One of these extracellular matrix macromolecules is fibronectin, a large glycoprotein found in plasma, in the extracellular matrix, and on the cell surface, which can influence cell-to-cell as well as cell-to-substratum adhesion.[6] Fibronectin is composed of 2 polypeptide chains, A and B, and contains binding sites for a number of substances. Integrins recognize multiple domains located on the fibronectin molecule. Arginyl-glycyl-aspartic acid (RGD), the "cell binding" domain, interacts with $\alpha_5\beta_1$, $\alpha_3\beta_1$, and other integrins. The alternately spliced connecting segment domain (CS-1), also involved in cell adhesion, is recognized by $\alpha_4\beta_1$.[7]

There is increasing evidence that surface proteoglycans, as well as integrins, are involved in the pathogenesis of the inflammatory process.[8] In this regard, a number of bioactive peptides from the 33-kD carboxyl terminal heparin binding domain from the A chain of fibronectin have been developed that inhibit RGD-independent binding of leukocytes to fibronectin by adhering to cell surface proteoglycans.[8-10] In addition, these peptides appear to inhibit adhesion of activated monocytes to cytokine-activated endothelial cells, which may play a role in the late influx of neutrophils.[11]

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The most potent of these synthetic fibronectin peptides reported in the literature is FN-C/H V.11 The synthetic fibronectin peptide (Trp-9-Tyr) has similar amino acid sequence as FN-C/H V, with the addition of a tyrosine residue (Figure 1).

We tested the hypothesis that inhibition of leukocyte adherence with Trp-9 Tyr would reduce the microvascular damage and progression of tissue necrosis following thermal injury.

METHODS

New Zealand White rabbits (3-5 kg; Myrtle's Rabbitry, Thompson Station, Ind) were shaved, and baseline cutaneous blood flow was measured with a laser Doppler blood-flow meter with a temperature controlled integrated probe (Perimed PF 4001, Stockholm, Sweden).[13] The animals were anesthetized with isoflurane inhalation (Abbott Laboratories, North Chicago, Ill). Twenty-four-gauge catheters (Becton-Dickinson, Sandy, Utah) were placed in ear veins. Three connected brass templates measuring 3 cm × 1 cm × 1 cm each with intervening 5 mm spaces were heated to 100°C and applied to the animals' backs for 30 seconds to create full thickness burns with intervening 5 mm zones of stasis.[14,15] Analgesia with 0.05 mg/kg of buprenorphine was administered every 12 hours (Reckitt & Colman Products Ltd., Hull, England). Blood flow measurements were obtained from the burn sites, the zones of stasis surrounding the burn site, and the unburned sites at 24, 48, and 72 hours postburn. The number of zones that progressed to necrosis was determined at 72 hours. Animals were killed at 72 hours with 100 mg/kg of intravenous pentobarbital (Abbott Laboratories, North Chicago, Ill).

Trp-9-Tyr is a 9 amino acid synthetic peptide of the fibronectin molecule (donated by Sentron Medical, Inc, Cincinnati, Ohio). It is stored in powder form at 4°C. Immediately before use, the powder is dissolved in normal saline to a 10-mg/ml solution for intravenous injection.

There were 3 experimental groups. Controls (n = 10) were given saline (1.0 ml/kg). The first treatment group (n = 10) was given the peptide, Trp-9-Tyr (5 mg/kg) immediately after the burn was produced, then 3, 6, 12, and 24 hours postburn. The second treatment group (n = 10) received Trp-9-Tyr immediately after the burn was produced, then every 6 hours for 48 hours postburn.

All animal experiments were approved by and performed in accordance with policies of the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston.

RESULTS

There were no significant differences in weight change following burn in the 3 groups. Gross visual observation and laser Doppler blood flow measurement of the burn sites were consistent with full-thickness injury. Average baseline laser Doppler blood flows of the 3 groups ranged from 70.69 to 80.56 perfusion units.

Bloodflow. Control animals had diminished blood flow in the zone of stasis at 24 hours postburn (29.97 ± 2.94). There was some recovery at 48 and 72 hours. However, blood flow remained significantly lower than baseline blood flow.

Animals in the 24-hour treatment group had significantly higher blood flow in the zone of stasis than did the controls at 24 hours postburn (42.25 ± 3.40). However, there was no difference in perfusion at 48 and 72 hours.

Skin perfusion of the animals treated for 48 hours was significantly higher than that of controls and of animals treated for 24 hours at all time points measured. Although blood flow was significantly lower than baseline at 24 hours postburn (56.23 ± 2.55), perfusion recovered and was not significantly different from baseline at either 48 or 72 hours (Figure 2).

Tissue Necrosis. Control animals had 16 (40%) of 40 zones of stasis that progressed to necrosis at 72 hours. Animals that received Trp-9-Tyr for 24 hours had 7 (17%) of 40 zones that progressed to necrosis, which is significantly less tissue destruction than the controls had ($P < .05$ χ^2). Animals treated for 48 hours had 4 (10%) of 40 zones that progressed to necrosis, which was also significantly less tissue loss than the controls had ($P < .05$ χ^2, Mann-Whitney test; Table 1). Photographs taken 72 hours after burns were produced demonstrate marginal zones with necrosis (Figures 3 and 4).

DISCUSSION

Emphasis on microvascular injury caused by leukocyte adherence to the endothelium has been the key to investigation of substances that inhibit inflammation. More recently, the extracellular matrix has become a point of interest for investigation of the inflammatory response to injury. Fibronectin, an extracellular matrix macromolecule, has reemerged as having a potentially significant role in the pathogenesis of the inflammatory processes.

Synthetic analogues of the RGD domain of fibronectin have been used to prevent acute and chronic experimental liver injury in mice. After

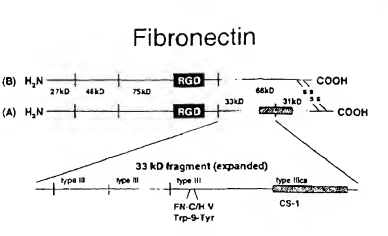

Figure 1. Diagram of location within fibronectin of peptide used in this study. *Boxed areas* indicate predicted active binding domain.[8,11,12]

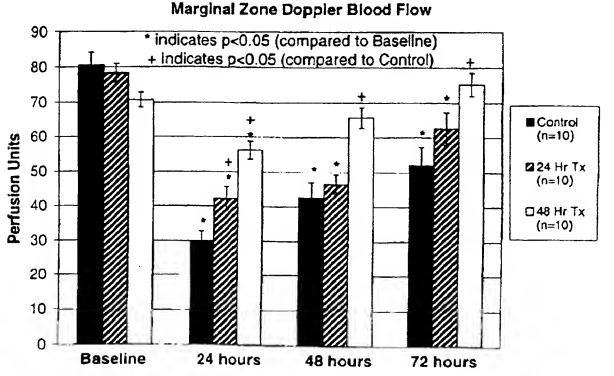

Figure 2. Blood flow in zones at baseline, 24, 48, and 72 hours: control, 80.56 ± 3.58, 29.97 ± 2.94, 42.67 ± 4.38, 52.17 ± 5.06; 24-hour treatment, 78.33 ± 2.64, 42.25 ± 3.40, 46.63 ± 2.69, 62.62 ± 4.69; 48-hour treatment, 70.69 ± 2.07, 56.23 ± 2.55, 65.70 ± 2.95, 75.19 ± 3.30. Laser doppler perfusion measurement in zones of stasis is presented as mean ± SEM for the control group *(solid bar)*, the 24 hour treatment group *(broken bar)*, and the 48 hour treatment group *(white bar)*. (*$P < .05$ vs baseline by t test; +$P < .05$ vs control by t test.) Control animals had significant decreases in perfusion in the zones of stasis at all postburn time points. Animals in the 24-hour treatment group had higher perfusion at 24 hours postburn than animals in the control group had. Animals in the 48-hour treatment group had higher blood flow in the zones of stasis at 24, 48, and 72 hours versus animals in the control group.

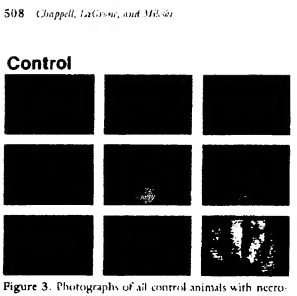

Figure 3. Photograph of all control animals with necrosis at 72 hours. Dark areas in the marginal zones of stasis are considered necrotic.

Table 1. Tabulation of zones of stasis progressing to necrosis comparing the 24-hour and 48 hour treatment groups with controls

	Number of Animals	Total Number of Zones	Zones with Necrosis
Control	10	40	16
24 hour	10	40	7
48 hour	10	40	4

For animals in the 24-hour treatment group, 7 (17%) of 40 zones progressed to necrosis, significantly fewer zones than for controls ($P < .05$ χ^2 test). Animals treated for 48 hours had necrosis in only 4 (10%) of 40 zones, significantly less tissue destruction than controls had ($P < .05$ χ^2 and Mann Whitney tests).

induction of hepatitis from intravenous concanavalin A injection, animals that received intravenous RGD mimetics had lower serum levels of liver enzymes and less liver damage by histology than did untreated mice.[16]

Synthetic fibronectin peptides derived from the 33-kD carboxyl-terminal heparin binding domain of fibronectin have demonstrated efficacy in blocking neutrophil accumulation in models of acute inflammation. Two of the more potent synthetic peptides identified thus far are FN-C/H V and CS-1. Using a rodent model of ischemic brain injury, Yanaka et al[17,18] demonstrated that administration of FN-C/H V and CS-1 decreased leukocyte accumulation, effectively reduced infarct size, and improved neurologic assessment. Synthetic fibronectin peptides block development of inflammatory lesions in the salivary glands of transforming growth factor-β_1 knock-out

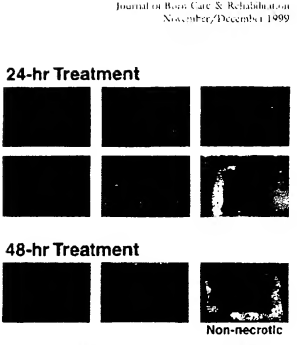

Figure 4. Peptide-treated animals with necrosis at 72 hours. Animals treated for 24 hours are shown in the upper panel, and the 2 animals treated for 48 hours are shown in the bottom panel. An example of burn injury with no surrounding necrosis in the marginal zones, labeled non-necrotic, is shown in the lower right hand corner.

mice and restore saliva production.[19] These peptides blocked leukocyte infiltration into heart and lung tissues of transforming growth factor-β_1 knock out mice, as demonstrated by histopathology. These animals had reduced weight loss and extended life span compared with untreated knock-out mice.[20]

As a cationic hydrophilic peptide, FN-C/H V is thought to adhere to cell-surface proteoglycans.[8,11] Cell-surface proteoglycans mediate a spectrum of cell-binding activities. FN-C/H V may alter proteoglycan interaction with selectins or integrin-dependent leukocyte homing.[8,20,21]

Migration of neutrophils into sites of inflammation has been reported to be initiated by a factor released by monocytes.[22] The synthetic fibronectin peptide FN C/H V has also been demonstrated to inhibit monocyte accumulation by means of the pathways related to CS-1.[11] CS-1 interacts with $\alpha_4\beta_1$ integrin expressed on monocytes and alters their function. This suggests that the synthetic fibronectin peptides may inhibit late accumulation of neutrophils into inflammatory tissue.

Other mechanisms of action for the peptide FN-C/H V have been considered. It may block signal transduction pathways and cytokine presentation. In addition, an $\alpha_4\beta_1$ integrin also serves as a receptor

for vascular cell adhesion molecule-1, which is expressed on endothelial cells.[11] By interacting with $\alpha_4\beta_1$ integrin, FN-C/H V may block leukocyte adhesion to the microvascular endothelium.

In this study, the administration of synthetic fibronectin peptide Trp-9-Tyr reduced tissue necrosis following burn injury. These results were somewhat evidenced in the 24-hour treatment group. However, the improvements in blood flow and tissue necrosis were significant in the 48 hour treatment group. Previous studies with this rabbit model have shown a correlation between histologic verification of tissue necrosis and gross visual evidence of necrosis as well as Doppler blood flow.[14,15] Because of our previous experience, histologic specimens were not taken in this study.

We hypothesize that the difference in blood flow between the 2 treatment groups seen at 24 hours was due to variation in the dosing schedules. Animals treated for 24 hours received Trp-9-Tyr immediately, then at 3, 6, 12 and 24 hours. The 48-hour treatment group received an 18-hour dose rather than a 3-hour dose. It is possible that the constant 6-hour dosing interval is more effective than an initial bolus of Trp-9-Tyr. Furthermore, the inflammatory process produces tissue destruction for a period longer than 24 hours. By extending the treatment beyond 24 hours, we were able to demonstrate improved blood flow and tissue salvage in the 48-hour group, compared with the 24-hour group.

For this study, we used a small burn model. The effect of Trp-9-Tyr on a larger burn injury would be difficult to predict because of the short half-life of the peptide. A larger burn would generate a greater inflammatory response and would possibly require higher dosing for the same effect.

One of the most significant concerns about the value of inhibiting leukocyte adherence is the possibility of increasing the susceptibility to infection. We used a New Zealand White rabbit model of infection to investigate this concern. Previous studies suggest that monoclonal antibodies to integrins and selectins, which block leukocyte adherence, significantly increase susceptibility to infection.[23] However, preliminary experiments using Trp-9-Tyr in our rabbit infection model indicate that the peptide does not increase infection when compared with saline controls.

In summary, intravenous administration of Trp-9-Tyr improved blood flow in the marginal zones of stasis surrounding a burn. The use of the synthetic fibronectin peptide was associated with less tisstue destruction following thermal injury.

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